Cloning and Identification of Highly Expressed Genes in Barley Lemma and Palea

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ABSTRACT

The lemma and palea (lemma/palea) of cereals are photosynthetic organs that supply the developing kernel with carbon and nitrogen. Because of their rigid structure, the lemma/palea can also protect the kernel from pathogens and herbivory. However, very little is known about specific gene expression that enabled the lemma/palea carry out their functions. We have constructed three subtracted cDNA libraries from lemma/palea of barley (*Hordeum vulgare* L. cv. Morex) at the elongation (between pollination and milky stages) through dough stages of kernel development. Differential screening and northern hybridization showed that the cloned genes were highly expressed in the lemma/palea, compared with the flag leaf. Thus, they contained unique sequences not found in the flag leaf or were expressed in the lemma/palea at much higher levels, appearing as if they were induced. Sequence analysis of 226 clones identified a high proportion of genes for defense, structure, amino acid biosynthesis, and photosynthesis. High expression levels of defense-related genes strongly suggest that lemma/palea constitutively accumulate defensive molecules to inhibit invasion of florets and kernels by pathogens. Increased expression of genes involved in cell wall synthesis and structural repair can improve physical barriers to herbivores and pathogens. High expression of genes for amino acid biosynthesis and photosynthesis indicates that the lemma/palea are major sources of nitrogen and carbon for the growing kernel.

Cereal inflorescences are organized into spikelets, and each spikelet contains one or more florets (Briggs, 1978). In turn, each floret consists of a pair of glumes, a lemma, a palea, a pair of lodicules, three stamens, and a pistil. The lemma bears a flower at its axil and, later, with the palea forms the husk (Fig. 1). The lemma/palea play indispensable roles in the development of cereal seeds. These photosynthetic organs, together with the flag leaf, and upper stem, supply florets and developing kernels with carbohydrates (reviewed in Duffus and Cochrane, 1993). The lemma/palea and other photosynthetic organs of the spike contribute up to 76% of kernel dry weight. Amino acids are the main source of nitrogen for the developing kernel, and a major portion of this may be contributed by the lemma/palea. In addition, being an outer cover, the lemma/palea may protect florets and kernels from attack by pathogens and insects. Very little study has been devoted to genes pertaining to the unique functions of these organs. Analysis of genes highly or uniquely expressed in the lemma/palea would provide molecular clues to the functions of these organs.

Loss-of-function studies and cloning of floral homeotic genes suggest that the lemma/palea of grasses are developmentally analogous to sepals (Bowman, 1997; Ambrose et al., 2000; Prasad et al., 2001). In maize (*Zea mays* L.), the male sterile mutant *silky1 (si1)*, encodes a MADS box gene orthologous to the *Arabidopsis AP3* (a B class organ identity gene; Ambrose et al., 2000). Mutations in the s*i1* locus result in the conversion of lodicules into lemma/palea. Since lodicules are the equivalent of petals (specified by class A and B genes), replacement of lodicules by lemma/palea in *si1* mutants indicates that lemma/palea are specified by the class A genes, as are the sepals (Ambrose et al., 2000). Direct evidence for the involvement of class A genes in the specification of lemma/palea is the cloning of cereal genes orthologous to the *Arabidopsis AP1*. The rice (*Oryza sativa* L.) *OsMADS1* gene is expressed early in the spikelet meristem, and later its expression is confined to the lemma/palea primordia (Prasad et al., 2001). This expression pattern is consistent with the early expression of *AP1* in *Arabidopsis* floral meristem and later in sepal primordia (Bowman et al., 1993; Parcy et al., 1998). *RAP1A*, also a rice ortholog of *AP1* gene, is expressed early in flower development; later its expression is confined to the developing lemma/palea and lodicules (Kyozuka et al., 2000).

Although sufficient evidence exists to show that floral homeotic genes specify lemma/palea, no study has been undertaken to determine which genes are active in mature lemma/palea. Expression analysis would enable us to identify genes that contribute to the functions of lemma/palea. Here we used the PCR-based suppression subtractive hybridization (SSH) method to identify genes overexpressed in mature lemma/palea (from elongation, between pollination and milky stages, to early dough stages of kernel development) relative to fully expanded flag leaf. We have identified highly expressed gene sets, which reflect the roles of lemma/palea in protecting and nourishing the developing kernel.

MATERIALS AND METHODS

Plant Material

Barley plants ('Morex') were grown in a greenhouse maintained at 16 to 21°C. Plants received supplemental lighting from sodium arc vapor lights for 16 h per day. For total RNA isolation, mature lemma/palea, and 4th, 5th, 6th, and flag leaves were collected. Lemma/palea were collected from spikes at three stages of kernel development: elongating (between pollination and milky stages), gelatinous and early dough (Skadsen et al., 2000). To avoid variations in gene expression because of differences in development, care was taken to collect leaves with similar stages of development to the lemma/palea.

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Construction of Subtracted cDNA Libraries

Total RNA was isolated from mature lemma/palea and the flag leaf with guanidinium thiocyanate (Chirgwin et al., 1979). Poly(A)$^+$ RNA was isolated using an oligo(dT) matrix. Suppression subtractive hybridization (SSH; Diatchenko et al., 1998) was performed to create forward and reverse subtracted cDNA libraries using the PCR-Select cDNA Subtraction kit (Clontech, Palo Alto, CA). The forward subtraction used tester cDNA obtained from the pooled lemma/palea mRNA and driver cDNA from the flag leaf. Driver cDNAs were the reference and were targets for elimination during subtraction. In the reverse subtraction, the tester cDNA was derived from the flag leaf, and pooled mRNA from the three stages of lemma/palea was used as driver. The reverse subtraction served as a control.

Two-microgram aliquots of poly(A)$^+$ mRNA from each tester and driver were used for cDNA synthesis. The double stranded cDNA was digested with *Rsa*I to increase the subtraction efficiency during subsequent hybridizations. Each digested tester cDNA was subdivided into two portions. One-half of the cDNA was ligated with adaptor 1 and the other half with adaptor 2R. Driver cDNA was not subjected to adaptor ligation. Denatured adaptor 1- or 2R-ligated tester cDNA was hybridized separately with an excess of denatured driver cDNA at 68°C for 8 h. Then the two primary hybridization samples were mixed together without denaturation and hybridized again with freshly denatured driver cDNA at 68°C for 24 h. After filling in the ends with DNA polymerase, the entire population of cDNA molecules was subjected to two rounds of PCR, the first using PCR primer 1 and the second using nested primers 1 and 2R.

Cloning the Subtracted Library

The forward-subtracted cDNA was cloned into pCR2.1-TOPO TA cloning vector (Invitrogen, Carlsbad, CA) or into *Sma*I-cut pBluescript II SK (Stratagene, La Jolla, CA). For cloning into pCR2.1-TOPO, the PCR reaction was incubated for a further 15 min at 72°C to ensure that the subtracted cDNA fragments contained 3′ A overhangs. For cloning into pBluescript II SK, PCR products were digested with *Rsa*I and purified with the Qiaquick PCR purification kit (Qiagen, Valencia, CA). Four microliters of the PCR amplified cDNA was ligated with 50 ng of vector. Two microliters of the ligation product was introduced into DH5α cells. The library was plated onto LB-agar plates containing 100 μg/L Ticillin, 40 μL of 20 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), and 40 μL of 100 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG).

Differential Screening

To identify genes that are highly expressed in the lemma/palea, the cDNA libraries were subjected to a differential screening procedure (PCR-Select Differential Screening Kit; Clontech, Palo Alto, CA). Recombinant colonies were randomly picked and grown in 3 mL LB medium containing 100 μg/L Ticillin. An aliquot of the culture was used to amplify inserts by PCR. The presence of a single PCR product was confirmed by agarose gel electrophoresis. PCR products were arrayed on Nytran N membranes (Schleicher & Schuell, Inc., Keene, NH) with Hybri-Dot Manifold (Life Technologies Inc., Gaithersburg, MD). Two identical membranes with cDNA clones (probes) arrayed in duplicate were prepared. Membranes were hybridized with ^{32}P-labeled target cDNA populations (10^6 cpm/mL activity) derived from the forward- and reverse-subtracted cDNA pools. Labeling target cDNA and hybridization were performed as described previously (Skadsen et al., 1995) except that hybridization and washing were done at 62°C. Clones representing mRNAs that are highly expressed hybridized only with the forward-subtracted cDNA. Clones that hybridized with the reverse-subtracted cDNA population were considered escapes and excluded.

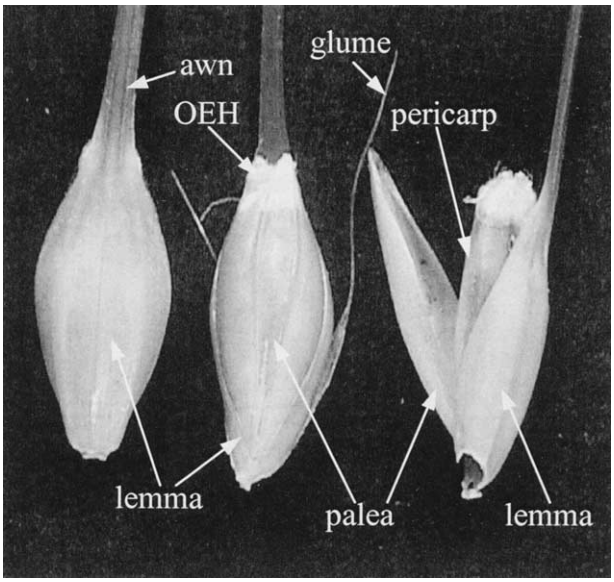

Fig. 1. Lemma and palea organs covering developing kernels of Morex barley. Left, lemma completely covers abaxial surface of kernel. The awn is continuous with the lemma. Center, palea covers most of the adaxial surface and is partially covered by the lemma. Ovary epithelial hairs (OEH) protrude above the palea tip. Right, lemma and palea teased apart from the developing kernel exposing the pericarp.

DNA Sequencing

Bacterial clones harboring cDNAs for differentially expressed genes were grown overnight in 3 mL LB, and plasmids were purified with the Qiaquick mini-prep kit (Qiagen, Valencia, CA). Single-pass sequencing of inserts was performed with universal forward (5′-CGCCAGGGTTTTCCCAGT CACGAC-3′) or reverse (5′-AGCGGATAACAATTTCA CACAGGA-3′) primers using the BigDye cycle sequencing mix (PerkinElmer Applied BioSystems, Foster City, CA) with the following PCR thermal profiles: an initial 95°C for 3 min, followed by 95°C for 20 s, 50°C for 30 s, 60°C for 4 min, and 72°C for 7 min for 35 cycles. Sequencing products were analyzed at the University of Wisconsin-Madison Automated DNA Sequencing Facility on ABI Model 377 and 3700 Automatic DNA Sequencers (PerkinElmer Applied BioSystems, Foster City, CA). Raw sequence data was edited by Chromas version 2.21 (Technelysium Pty. Ltd., Australia) and the online VecScreen program available from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov; verified 15 January 2004). EST identities and potential functions were determined by sequence comparison to the nonredundant GenBank database by BLASTN (BLAST 2.2.1) with default parameters (Altschul et al., 1997). In instances where an unannotated match was obtained, further searches of the protein database were conducted by BLASTX, and sequence homology information was used to assign putative identities (Table 1).

Northern Blot Analysis

Ten micrograms of total RNA from each organ was separated on 1.2% (w/v) agarose-formaldehyde gels and trans-

Table 1. A partial list of cDNA clones representing highly expressed genes in the lemma/palea of barley, relative to the flag leaf. Genes were grouped into functional categories, although some genes may have multiple functions. Some genes were represented by more than one clone. To avoid redundancy, only single clones are presented for each gene. The first number under clone names indicates the developmental stage at which lemma/palea were collected (1-, elongation; 2-, gelatinous; 3-, dough kernel stage). For clones with matches in the GenBank databases, E-values and percent identity are shown.

Clone	GenBank Acc. No.	Homologous GenBank sequences and accession numbers	E-value	% identity
		Defense		
1-5	BM928832	*H. vulgare* jacalin-like protein LEM2, AY092765	0.00	99
1-14	BM928838	*Z. mays* glutathione S-transferase, AF244682	8e-11	82
1-25	BM928870	*H. vulgare* glutathione peroxidase, AJ238745	9e-64	99
1-57/1	BQ135337	*H. vulgare* jacalin-like protein LEM2, AY092765	5e-39	100
1-58	BQ135339	*A. thaliana* UVB-resistance protein, AAD43920	7e-61	78
1-107	BQ135351	*H. vulgare* lipid transfer protein 7a2b, X96979	e-154	98
1-116	BQ135353	*O. sativa* putative peroxidase, BAB12033	2e-11	87
1-247	BQ294519	*H. vulgare* gene encoding jasmonate-induced protein, X98124	e-92	95
1-249	BQ294521	*H. vulgare* mRNA for allene oxide synthase, AJ250864	9e-96	100
1-259	BQ294524	*H. vulgare blt14* mRNA, X57554	4e-95	98
1-262	BQ294525	*A. tauschii* glutathione-S-transferase 2, AAG40562	4e-33	75
1-318	CD240717	*H. vulgare* endo-1,4-beta-glucanase Cel1, AB040769	e-144	100
2-36	BQ134700	*A. thaliana* ankyrin-like protein, NP_201208	2e-75	78
2-73	BQ294532	*N. tabacum* NADPH oxidase, CAC84140	0.00	98
2-134	BQ294538	*H. vulgare* jacalin-like protein LEM2, AY092765	5e-39	100
2-150	BQ294541	*H. vulgare Bsi1* gene for putative protease inhibitor, Z48729	e-129	97
2-163	BQ294542	*O. sativa* OsABA2 mRNA for zeaxanthin epoxidase, AB050884	e-146	88
3-3	BM928860	*H. vulgare* catalase 2 (Cat2), U20778	4e-49	80
3-55	BQ134659	*A. thaliana* disease resistance-like protein, AAG26078	3e-42	57
3-104	BQ134669	*T. aestivum* proline-rich protein, X52472	e-144	95
3-125	CD240765	*H. vulgare* oxalate oxidase, HVY14203	e-117	88
3-167	BQ134686	*A. thaliana* NBS/LRR disease resistance protein (RFL1), CAB80047	6e-36	93
3-174	BQ134688	*H. vulgare* methyljasmonate-inducible lipoxygenase, U56406	e-107	98
3-189	BQ134692	*H. vulgare* cold acclimation WCOR413-like protein gamma form, AF465840	e-139	93
		Structural		
1-4	BM828831	*T. aestivum* β-tubulin 3, U76746	e-118	95
1-17	BM928839	*H. vulgare* Arabinoxylan arabinofuranohydrolase, AF320325	e-154	99
1-54	BQ135334	*A. thaliana* UDP-glucose dehydrogenase, AF424576	2e-40	81
1-256	BQ294531	*H. vulgare* mRNA for α-tubulin 4 (atub4 gene), AJ276012	4e-43	98
2-6	BM928850	*T. aestivum* proline-rich protein, X52472	e-131	91
2-8	BM928851	*Z. mays* caffeoyl CoA *O*-methyl transferase, AJ242980	3e-40	92
2-63	BQ134708	*H. vulgare* α-tubulin, U40042	e-132	99
2-78	BQ294533	*H. vulgare S*-adenosylmethionine synthetase, D63835	e-148	99
2-84	BQ294534	*H. vulgare* paf93 gene homologous to Cor47, X84056	0.00	98
3-42	BQ134656	*A. thaliana* putative actin depolymerizing factor 1, AY062940	3e-18	83
3-153	BQ134680	*O. sativa* putative *O*-methyltransferase ZRP4, AAL31646	3e-14	92
3-188	BQ134691	*H. vulgare* CM 72 germin/oxalate oxidase subunit, U01963	e-134	90
		Amino acid biosynthesis		
1-11	BM928837	*H. vulgare* alanine aminotransferase, Z26322	e-142	93
1-56	BQ135336	*H. vulgare* glutamine-dependent asparagine synthetase, AF307145	0.00	99
1-167	BQ135357	*Z. mays* lysine-ketoglutarate reductase/saccharopine dehydrogenase, AF003551	2e-72	84
2-35	BQ134699	*Hordeum* sp. x Triticum sp. glycine decarboxylase P subunit, AF024589	5e-48	75
2-38	BQ134702	*T. aestivum* Δ^1–pyrroline-5-carboxylate synthetase, AF022914	2e-46	83
2-57	BQ134707	*O. sativa* Fd-dependent glutamate synthase (Fd-GOGAT), Y12595	e-80	88
2-67/1	BQ134710	*A. thaliana* putative chorismate mutase/chloroplast prephenate dehydratase, NP_180350	2e-38	91
2-67/2	BQ134711	*O. sativa spe1* for arginine decarboxylase, X56802.1	2e-22	89
2-331	CD240757	*A. thaliana* anthranilate phosphoribosyltransferase, T04208	4e-58	85
3-120	BQ134672	*A. thaliana* branched chain alpha-keto acid dehydrogenase E2 subunit (dihydrolipoyl transacylase), AAF35280	2e-46	76
3-168	BQ134687	*H. vulgare* phenylalanine ammonia-lyase, Accession No. Z49146	e-119	100
		Photosynthesis		
1-10	BM928836	*T. aestivum* rbcS, X00235	e-118	96
1-73	BQ135342	*O. sativa* putative chloroplast inner envelope protein, AAG13554	e-17	74
1-91	BQ135346	*O. sativa* NADP-dependent malic enzyme (NADP-ME), D16499	e-129	87
2-22	BM928854	*H. vulgare* rbcL, X00630	e-114	96
2-43	BQ134703	*H. vulgare* photosystem I antenna protein, X84308	6e-97	100
2-86	BQ294535	*N. tabacum* 37 kDa chloroplast inner envelope membrane protein, CAA64422	e-53	78
2-185	BQ294545	*T. aestivum petF* gene for ferredoxin, X75089	e-155	97
3-131	BQ134673	*T. aestivum* oxygen evolving PS II protein (psbP gene), X57407	e-113	94
3-133	BQ134674	*O. sativa* glutaredoxin (thioltransferase), X77150	8e-34	84
3-146	BQ134677	*O. sativa* Chl a/b-binding Cab2R gene, X13909	e-48	94
		Protein synthesis and processing		
1-2	BM928830	*P. sativum* chloroplast L1-like ribosomal protein, X82776	7e-22	86
1-74	BQ135343	*O. sativa* 60S ribosomal protein L18, PID: 14595709	5e-83	92
1-104	BQ135350	*A. thaliana* putative tyrosyl-tRNA synthetase, AY075664.1	2e-18	79
1-110	BQ135352	*O. sativa* 26S proteasome regulatory particle non-ATPase subunit 12, AB037153	e-28	81
1-201	BQ294509	*O. sativa OsPBC1* mRNA for beta 3 subunit of 20S proteasome, AB026565	e-66	87
1-242	BQ294517	*H. vulgare* mRNA for elongation factor 1-alpha, Z50789	0.00	99
1-292	BQ294562	*T. aestivum* VDAC3 mRNA for voltage dependent anion channel, X82148	0.00	96

Continued next page.

Table 1. Continued.

Clone	GenBank Acc. No.	Homologous GenBank sequences and accession numbers	E-value	% identity
1-293	BQ294563	*T. aestivum WC3* mRNA for cysteine proteinase inhibitor, AB038394	3e-51	89
2-13	BQ134695	*H. vulgare* ribosomal L41 protein, AJ001160	e-171	95
2-16	BQ134697	*Glycine max* ubiquitin carrier protein 4, AAF03236	9e-15	92
2-37	BQ134701	*A. thaliana* 60S ribosomal protein L36, AY060496	2e-26	86
2-45	BQ134704	*Z. mays* epsilon-COP protein (coatomer), AF216853	2e-40	88
2-50	BQ134705	*T. aestivum* 70 kDa heat shock protein, AF005993	4e-71	88
2-145	BQ294540	*O. sativa* mRNA for ribosomal protein L35 (NH77 gene), D10407	e-25	87
2-206	BQ294549	*A. thaliana* putative 60S ribosomal protein L4/L1(RPL4A), NP_187574	2e-36	77
2-279	BQ294557	*H. vulgare* mRNA for plasma membrane H^+–ATPase (*ha1* gene), AJ344078	0.00	99
2-283	BQ294560	*O. sativa* mRNA for ribosomal protein S31, D38011	7e-53	86
2-312	CD240748	*A. thaliana* 40S ribosomal protein S25, NP_179752	e-15	94
2-350	CD240763	*S. bakko* protein translation factor SUI1 homolog, BAA24697	2e-14	97
3-4	BM928861	*Z. mays* yptm2 gene(GTP-binding protein), X63278	e-118	95
3-6	BQ134650	*C. sativa* 26S proteasome regulatory subunit S12 isolog-like protein, AF417302	e-13	80
3-7	BQ134651	*H. vulgare* elongation factor 1 alpha (EF-1α), L11740	e-136	100
3-21	CD240764	*A. thaliana* AtRer1A mRNA, AY044321	3e-17	81
3-47	BQ134657	*B. vulgaris* elongation factor 2, Z97178	4e-70	83
3-77	BQ134666	*Z. mays* ribosomal protein S8, U64436	2e-14	83
3-83	BQ134714	*A. thaliana* ubiquitin-like protein, AY072534	7e-05	83
3-116	BQ134671	*O. sativa* polyubiquitin (RUBQ2)gene, AF184280	0.00	90
3-144	BQ134676	*S. bakko* protein translation factor SUI1 homolog, O48650	2e-14	97
3-164	BQ134685	*Z. mays* cysteine proteinase 1 precursor, Q10716	2e-74	57
3-180	BQ134690	*T. aestivum* HSP80-2 protein, Accession No. X98582	0.00	94
3-190	BQ134693	*O. sativa* peptide transporter, BAB62326	e-37	86
		General metabolism		
1-24	BM928842	*Z. mays* adenosine kinase, AJ012281	0.00	88
1-30	BM928843	*H. vulgare* cytosolic GA3PDH, M36650	e-90	98
1-63	BQ135341	*O. sativa* putative EREBP-type transcription factor, AF364176.1	3e-07	81
1-78	BQ135344	*Z. aethiopica* geranylgeranyl reductase, AF055296	5e-50	88
1-86	BQ135345	*A. thaliana* transcriptional coactivator-like protein, PID: 15238570	e-30	81
1-93	BQ135347	*O. sativa* methylmalonate semi-aldehyde dehydrogenase, AF045770	0.00	87
1-179	BQ135359	*T. turgidum* ADP/ATP carrier, X80023	0.00	95
1-233	BQ294515	*A. thaliana* succinic semi-aldehyde dehydrogenase (*gabD*), AY056147	0.03	77
1-254	BQ294522	*O. sativa* putative G-box binding protein mRNA, AF466286	3e-48	86
1-289	BQ294529	*Z. mays* histone deacetylase HDA110 isoform 4, AAM93215	4e-37	71
1-313	CD240714	*T. aestivum* cell wall invertase (IVR3) mRNA, AF030421	0.00	94
1-334	CD240726	*O. sativa* putative ABC transporter, PID: BAB68118	e-30	96
2-1	BQ134694	*A. thaliana* putative mitochondrial dicarboxylate carrier protein, AAD22351	3e-19	94
2-27	BQ134698	*H. vulgare* hexaprenyl dihydroxybenzoate methyl transferase, BM817329	e-154	98
2-231	CD240752	*H. vulgare* mRNA for 14-3-3 protein homolog (14-3-3a), X62388	0.00	99
2-287	CD240740	*O. sativa* mRNA for ADP-ribosylation factor, D17760	2e-95	91
2-316	CD240751	*T. aestivum* calmodulin TaCaM1-2 mRNA, U48688	e-158	95
2-328	CD240756	*O. sativa* ferritin, PID: AF519570	4e-22	94
2-2800	CD240736	*T. aestivum* calmodulin TaCaM1-3 mRNA, U48689	e-89	96
3-23	BM928868	*Tritordeum S*-adenosyl methionine decarboxylase, X83881	e-103	92
3-141	BQ134675	*C. annuum* GTP-binding protein, AF108896	7e-16	84
3-158	BQ134682	*T. aestivum S*-adenosyl-L-homocysteine hydrolase, L11872	3e-55	96
3-159	BQ134683	*A. thaliana* glycosyl hydrolase family 1, NP_176375	2e-26	75
		Unknown functions		
1-7	BM928834	*H. vulgare* EST, BF631545	0.00	99
1-9	BM928835	*H. vulgare* EST, AW983427	0.00	98
1-19	BM928840	*H. vulgare* EST, AW982342	e-123	97
1-19/1	BQ135327	*H. vulgare* EST, AW982342	e-128	99
1-19/2	BM928841	*T. aestivum* EST, BM138480	e-102	93
1-20	BQ135328	*H. vulgare* EST, AL500360	e-118	98
1-28	BQ135329	*H. vulgare* EST, BG 344478	0.00	99
1-34	BQ135330	*H. vulgare* EST, AV928554	0.00	99
1-37	BM928844	*H. vulgare* EST, BE455883	e-124	93
1-43	BQ135331	*H. vulgare* EST, BF267321	3e-87	98
1-45	BQ135332	*H. vulgare* EST, AJ435154	0.00	100
1-50	BM928845	*H. vulgare* EST, AV835724	2e-94	96
1-51	BQ135333	*H. vulgare* 23 KD jasmonate-induced protein, P32024	e-29	100
1-52	BM928846	*H. vulgare* EST, BE602255	6e-75	96
1-57/2	BQ135338	*H. vulgare* EST, BF627351	e-160	99
1-59	BQ135340	*H. vulgare* EST, AJ432920	1e-90	100
1-97	BQ135348	*O. sativa* genomic DNA, chromosome 4, BAC clone AL606454	4e-65	85
1-99	BQ135349	*T. aestivum* EST, BJ238105	0.00	96
1-158	BQ135354	*H. vulgare* EST, BG366909	2e-63	100
1-159	BQ135355	*H. vulgare* EST, AV946206	e-146	99
1-176	BQ135358	*H. vulgare* EST, AJ228933	e-114	96
1-191	BQ294507	*H. vulgare* EST, BG418158	e-112	89
1-196	BQ294508	*H. vulgare* EST, BI958561	e-104	98
1-202	BQ294510	*H. vulgare* EST, BE437535	e-138	100
1-218	BQ294511	*H. vulgare* EST, BG344942	e-128	96

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Table 1. Continued.

Clone	GenBank Acc. No.	Homologous GenBank sequences and accession numbers	E-value	% identity
1-226	BQ294513	*H. vulgare* EST, AJ000242	0.00	99
1-231	BQ294514	*H. vulgare* EST, BE437535	e-138	100
1-237	BQ294516	*H. vulgare* EST, BJ220905	3e-80	88
1-243	BQ294518	*H. vulgare* EST, BG366909	4e-59	99
1-248	BQ294520	*H. vulgare* EST, BG344942	e-144	100
1-283	BQ294526	*H. vulgare* EST, BJ320328	9e-89	94
1-285	BQ294527	*H. vulgare* EST, AJ435369	e-179	99
1-288	BQ294528	*H. vulgare* EST, AV946654	e-154	98
1-290	BQ294530	*H. vulgare* EST, BI779037	4e-85	99
1-296	BQ294531	*H. vulgare* EST, BM817329	e-154	98
1-301	CD240710	*H. vulgare* EST, BJ476948	5e-68	99
1-302	CD240711	*H. vulgare* EST, BJ449363	e-155	100
1-304	CD240712	*H. vulgare* EST, BQ463395	2e-71	100
1-305	CD240713	*H. vulgare* EST, AV935179	e-130	99
1-317	CD240716	*H. vulgare* EST, BJ458830	e-126	96
1-321	CD240718	*H. vulgare* EST, AV912609	6e-73	98
1-322	CD240719	*H. vulgare* EST, AV935179	e-133	100
1-325	CD240720	*H. vulgare* EST, BJ456827	e-156	100
1-327	CD240721	*H. vulgare* EST, BF618065	e-84	100
1-331	CD240723	*H. vulgare* EST, BF631514	e-161	100
1-332	CD240724	*H. vulgare* EST, AV914260	0.00	100
1-333	CD240725	*H. vulgare* EST, BJ484241	7e-79	98
1-337	CD240727	*H. vulgare* EST, BQ471537	e-160	100
1-340	CD240728	*H. vulgare* EST, AJ466811	4e-96	100
1-350	CD240729	*H. vulgare* EST, AV923903	e-140	98
1-351	CD240730	*H. vulgare* EST, BJ484074	0.00	99
1-354	CD240731	*H. vulgare* EST, BQ464082	0.00	99
1-355	CD240732	*H. vulgare* EST, BI776443	e-160	98
1-357	CD240733	*H. vulgare* EST, BJ484074	0.00	99
1-365	CD240735	*H. vulgare* EST, BQ468605	e-129	100
2-2	BM928848	*H. vulgare* EST, BG416709	0.00	96
2-20	BM928852	*H. vulgare* EST, BE519738	e-119	98
2-56	BQ134706	*H. vulgare* EST, BM816970	0.00	98
2-65	BQ134709	*H. vulgare* EST, AL511061	e-129	99
2-68	BQ134712	*H. vulgare* EST, AJ435174	e-139	100
2-102	BQ294536	*H. vulgare* EST, BI780480	e-120	98
2-123	BQ294537	*H. vulgare* EST, BF258493	e-81	92
2-142	BQ294539	*H. vulgare* EST, AL506963	0.00	99
2-181	BQ294544	*H. vulgare* EST, BF254980	e-173	97
2-188	BQ294546	*H. vulgare* EST, BF621961	e-180	99
2-195	BQ294547	*H. vulgare* EST, BG344593	e-146	93
2-203	BQ294547	*H. vulgare* EST, BM816504	4e-85	100
2-215	BQ294550	*H. vulgare* EST, AL502338	0.00	99
2-243	BQ294552	*H. vulgare* EST, AV938888	0.00	100
2-253	BQ294553	*H. vulgare* EST, AL510194	e-109	100
2-267	BQ294554	*H. vulgare* EST, BE438741	0.00	99
2-273	BQ294555	*H. vulgare* EST, AV909331	e-112	100
2-274	BQ294556	*H. vulgare* EST, BE405589	0.00	95
2-282	BQ294559	*H. vulgare* EST, BM098889	e-110	97
2-284	BQ294561	*H. vulgare* EST, AV918257	e-140	99
2-295	CD240741	*H. vulgare* EST, BJ483828	e-158	99
2-299	CD240742	*H. vulgare* EST, BJ468425	e-174	94
2-300	CD240743	*H. vulgare* EST, BQ461362	e-109	99
2-302	CD240744	*A. thaliana* leucine rich repeat protein family, NP_568696.1	2e-45	77
2-308	CD240746	*H. vulgare* EST, BQ461362	e-117	100
2-310	CD240747	*H. vulgare* EST, BF064925	2e-74	98
2-313	CD240749	*Z. mays* EST, AY103851	2e-36	88
2-325	CD240754	*H. vulgare* EST, BJ456308	4e-90	99
2-326	CD240755	*H. vulgare* EST, BQ461514	3e-81	98
2-333	CD240758	*H. vulgare* EST, BG417654	0.00	97
2-341	CD240759	*H. vulgare* EST, BE437544	e-114	97
2-344	CD240761	*H. vulgare* EST, BJ483828	e-160	99
2-2810	CD240737	*Z. mays* EST, AY104430	3e-73	87
2-2830	CD240738	*H. vulgare* EST AV936761	0.00	99
3-1	BM928859	*H. vulgare* EST, BF618065	e-104	97
3-9	BM928863	*H. vulgare* EST, AV832724	5e-67	91
3-10	BQ134652	*H. vulgare* EST, AJ433163	e-108	100
3-19	BM928866	*H. vulgare* EST, BF620670	e-149	97
3-20	BM928867	*H. vulgare* EST, AV836782	3e-93	98
3-26	BM928869	*H. vulgare* EST, AL507776	e-96	100
3-38	BQ134655	*S. propinquum* EST, BG487930	3e-44	87
3-52	BQ134658	*H. vulgare* EST, BG366909	5e-80	97
3-58	BQ134660	*H. vulgare* EST, BE421376	e-100	91
3-59	BQ134661	*H. vulgare* EST, BF259436	8e-25	84
3-64	BQ134662	*H. vulgare* EST, BG366909	6e-76	97
3-65	BQ134663	*H. vulgare* EST, BE193861	e-180	100

Continued next page.

Table 1. Continued.

Clone	GenBank Acc. No.	Homologous GenBank sequences and accession numbers	E-value	% identity
3-71	BQ134664	*H. vulgare* EST, AV935705	e-106	99
3-91	BQ134667	*H. vulgare* EST, BM816094	e-143	99
3-108	BQ134670	*H. vulgare* EST, AV911062	e-152	99
3-149	BQ134678	*H. vulgare* EST, AJ435335	3e-54	99
3-176	BQ134689	*H. vulgare* EST, AL502875	7e-70	99
		No match		
1-166	BQ135356			
1-314	CD240715			
1-359	CD240734			
2-24	BM928855			
2-314	CD240750			
2-323	CD240753			
3-13	BM928864			
3-17	BM928865			
3-151	BQ134679			

ferred to nylon membranes using 10× SSC (1× SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate, pH 7.0). Probes were prepared by amplifying cDNA inserts by PCR. Probes were labeled with [α-^{32}P]dCTP by random primer labeling and hybridized to blots at 62°C as described for differential screening.

RESULTS

Screening Libraries by Filter Arrays

With the suppression subtractive hybridization (SSH) method we generated three subtracted cDNA libraries of lemma/palea from the elongating, gelatinous, and early dough stages of kernel development. The cDNAs that contained lemma/palea-specific (differentially expressed) transcripts served as a tester, and flag leaf cDNA was used as a driver (reference). In parallel, a reverse-subtracted library was prepared with the flag leaf cDNA as a tester and the pooled lemma/palea cDNA as a driver. About 300 clones were obtained from each of the three subtracted lemma/palea libraries. The sizes of the cloned cDNAs ranged from 100 to 1200 bp. To prioritize suitable candidate clones for further characterization, we first screened libraries using nylon filter arrays. Duplicate arrays of individual PCR-generated inserts (probes) were prepared from randomly selected clones and hybridized with subtracted lemma/palea and flag leaf cDNA populations. Clones showing the greatest difference in hybridization signal intensity for the two cDNA populations represent mRNA sequences that are either unique to the target tissue (lemma/palea) or occur in vast excess relative to levels in the control tissue (flag leaf). Accordingly, lemma/palea- and flag leaf-enriched probes gave differing patterns of hybridization. The strongest signal was obtained with lemma/palea-enriched cDNA probes and the weakest signal was with the flag leaf-enriched probes (Fig. 2). Clones that gave the strongest signal with the lemma/palea-enriched cDNA represent genes that are highly expressed in the lemma/palea.

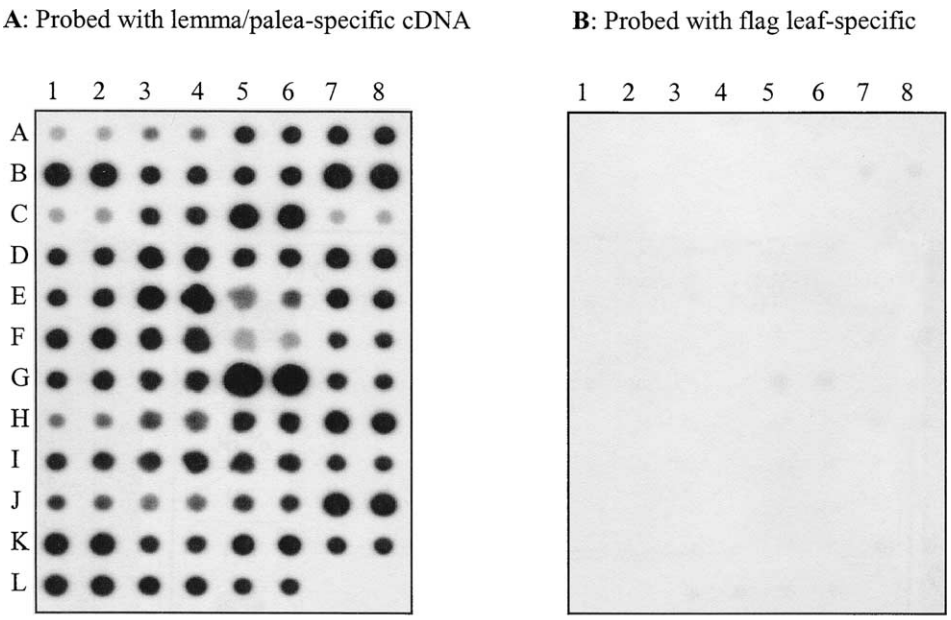
Genes for Defense, Structure, Amino Acid Biosynthesis, and Photosynthesis are Highly Expressed in the Lemma/Palea

We have sequenced 357 clones, chosen at random, from the three lemma/palea-specific libraries. A search to the GenBank databases revealed that 131 were redundant, and the remaining 226 represent single clones. Analysis of BLAST results for the 226 clones showed (i) 111 genes (49%) with known functions, (ii) 106 ESTs (47%) with matches in the GenBank database but of unknown functions, and (iii) 9 (4%) with no matches. Genes with known functions were grouped into functional clusters (Table 1), including defense, structure, amino acid biosynthesis, photosynthesis, turnover of proteins, and general metabolism. Most clones with unknown functions matched barley ESTs in the public database. The sequences generated in our study have been deposited in the GenBank database and can be browsed from the NCBI website (http://www.ncbi.nlm.nih.gov) under the accession numbers given in Table 1 or using the keyword "lemma and palea."

Confirmation of Differential Gene Expression by Northern Analyses

Northern analysis was used to confirm high expression levels of candidate genes in the lemma/palea. Among the 226 lemma/palea clones, six (1-5, 1-25, 1-52, 2-6, 2-24, and 3-13) were further tested by northern blotting. All were highly expressed in the lemma/palea, compared with the flag leaf, and showed various temporal expression patterns (Fig. 3). Genes 1-5 and 1-25 declined in expression as the lemma/palea matured. Expression of 1-52 and 2-6 peaked at the gelatinous stage and declined in the dough stage. Expression of 3-13 peaked in the dough stage, while 2-24 was uniformly expressed in lemma/palea from all developmental stages.

We were also interested to know expression patterns of the cloned genes in the lemma/palea vs. other leaves. Total RNA from the lemma/palea (in the gelatinous stage of kernel development), and from fully expanded 4th (from bottom), 5th, 6th (next to the flag leaf), and flag leaves was blotted onto nylon membranes. Blots were hybridized with ^{32}P-labeled 1-5, 1-14, 1-25, 1-52, 1-56, 1-107, 2-6, and 2-24 probes. As shown in Fig. 4, all genes were expressed more in the lemma/palea than in the leaves. Expression patterns among the leaves were spatially dependent. Genes 1-25, 1-56, and 2-6 were expressed equally in all leaves. Genes 1-52 and 1-107 were more abundant in the 4th leaf, but were expressed



	1	2	3	4	5	6	7	8
A	1-1	1-1	1-2	1-2	1-4	1-4	1-5	1-5
B	1-6	1-6	1-7	1-7	1-9	1-9	1-10	1-10
C	1-13	1-13	1-14	1-14	1-15	1-15	1-16	1-16
D	1-17	1-17	1-18	1-18	1-19	1-19	1-20	1-20
E	1-21	1-21	1-22	1-22	1-23	1-23	1-24	1-24
F	1-25	1-25	1-26	1-26	1-27	1-27	1-28	1-28
G	1-29	1-29	1-30	1-30	1-31	1-31	1-32	1-32
H	1-33	1-33	1-34	1-34	1-36	1-36	1-37	1-37
I	1-40	1-40	1-41	1-41	1-43	1-43	1-44	1-44
J	1-46	1-46	1-49	1-49	1-50	1-50	1-52	1-52
K	1-53	1-53	1-54	1-54	1-55	1-55	1-56	1-56
L	1-57/1	1-57/1	1-58	1-58	1-59	1-59	----	----

Fig. 2. Differential screening of a subtracted lemma/palea cDNA library from elongating stage. Duplicate filter arrays of random clones (probes) from the elongating stage lemma/palea library were hybridized with ^{32}P-labeled cDNA populations of 10^6 cpm/mL activity made from (A) forward subtracted cDNA (lemma/palea, tester; flag leaf, driver) and (B) reverse subtracted cDNA (flag leaf, tester; lemma/palea, driver). Clones in panel A showed differential hybridization and therefore represent mRNA sequences for genes highly expressed in the lemma/palea. The position of clones on the filter arrays is depicted in the table below.

uniformly in the flag, 5th, and 6th leaves. Transcripts for gene 1-5 accumulated more in the flag leaf than in the other leaves. Transcripts for gene 1-14 were abundant in the flag leaf and 4th leaves relative to the 5th and 6th leaves. Gene 2-24 was expressed higher in the 5th and 6th leaves than in the flag and 4th leaves.

DISCUSSION

The lemma/palea of grasses are modified leaves. Molecular and genetic studies suggest that the lemma/palea are developmentally analogous to the sepals of dicot flowers and are specified by class A genes (Bowman, 1997; Ambrose et al., 2000; Prasad et al., 2001). However, there is very little information about the genes that maintain the function of mature lemma/palea. It is not clear whether floral homeotic genes have prolonged effect on the function of lemma/palea or whether new sets of genes are activated. To identify genes involved in the maintenance of the lemma/palea functions, we have synthesized subtracted cDNA libraries using the suppression subtractive hybridization (SSH) method.

The lemma/palea are photosynthetic organs and can supply florets and developing kernels with photoassimilates. The lemma/palea are fibrous structures. By acting as covers they can prevent pathogens and pests from getting access to florets and developing kernels. Genes required for the lemma/palea to carry out these functions were represented in our SSH library (Table 1). High expression of genes for photosynthesis (*rbcS, rbcL*, photosystem I antenna protein, oxygen evolving photosystem II protein, glutaredoxin, ferredoxin, chl a/b-binding protein, and chloroplast inner envelope protein) and reactive oxygen species (ROS) scavenging (zeaxanthin epoxidase, glutathione S-reductase, glutathione peroxidase, and catalase) in the lemma/palea is an indication that these organs have a strong photosynthetic activity and require protection from ROS produced during the

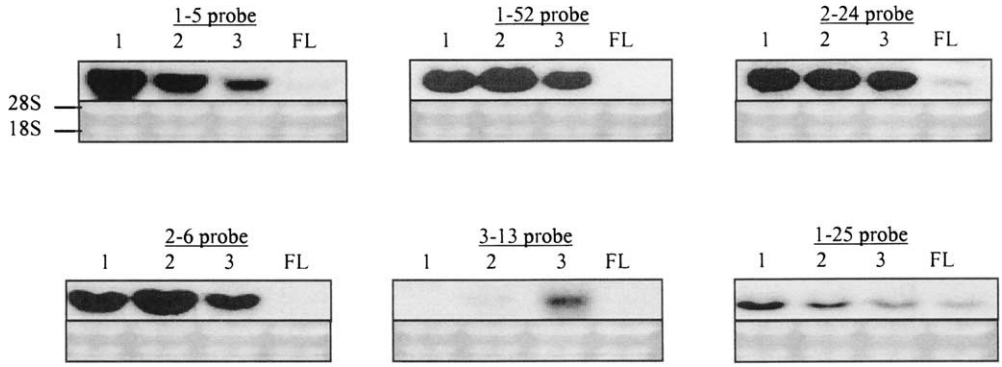


Fig. 3. Northern analysis of randomly selected clones confirms high expression levels in the lemma/palea compared with the flag leaf. Lemma and palea from the elongation, gelatinous and dough kernel stages were included to evaluate temporal expression patterns. Each lane contained 10 μg total RNA. The RNA blot was hybridized with ^{32}P-labeled probes 1-5, 1-25, 1-52, 2-6, 2-24 and 3-13. EtBr stained agarose-formaldehyde gel is included at the bottom of each figure to show equal loading. 1, 2, and 3 represent pooled lemma/palea RNA from elongation, gelatinous and early dough stages of kernel development, respectively; FL, flag leaf.

light reaction of photosynthesis. Furthermore, although the leaf and the stem are additional sources, relatively higher expression of photosynthesis genes in the lemma and palea suggests that these organs are a major supplier of photoassimilates for the developing kernel. Photosynthesis in the lemma, palea, awns and other organs of the barley spike account for up to 76% of the grain dry weight (Duffus and Cochrane, 1993).

The highly energetic light reaction and the involvement of oxygen make photosynthesis a rich source of ROS (Allen, 1995; Smirnoff, 1998). As active photosynthetic organs, the lemma/palea are vulnerable to damage by ROS, such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$). These ROS are generated at three sites during photosynthesis: the light harvesting complex (LHC), photosystem II reaction center and the photosystem I acceptor side (Niyogi, 1999). ROS are very reactive to lipids, proteins, and DNA (McKersie and Leshem, 1994). If unchecked, they can damage the photosynthesis apparatus and decrease the rate and efficiency of photosynthesis. Plants have enzymatic and nonenzymatic mechanisms to reduce the effects of ROS (Bohnert and Sheveleva, 1998; Smirnoff, 1998). Enzymes in the xanthophyll cycle (such as zeaxanthin epoxidase, clone 2-163) play an important role in the turnover of xanthophyll, which accepts excitation energy from the triplet chlorophyll, thereby preventing $^1O_2$ formation. Glutathione peroxidase, glutathione S-transferase and catalase detoxify H_2O_2 (Smirnoff, 1998; Roxas et al., 2000), and their expression in the lemma and palea should protect the photosynthesis apparatus. Glutathione peroxidase and glutathione S-transferase are chloroplastic (Roxas et al., 2000) and can scavenge H_2O_2 produced during photosynthesis. The accompanying H_2O_2 produced in peroxisomes by glycolate oxidase is neutralized by the peroxisomal enzyme catalase (CAT2). Ziegler-Jones (1989) suggested that the lemma/palea have a photosynthesis mechanism intermediate between the C3 and C4 pathways. Overexpression of a NADP-dependent malic enzyme (clone 1-91) in the lemma/palea is an indication that the lemma and palea are capable of C4 photosynthesis. However, high expression of glycine decarboxylase (clone 2-35), which catalyzes the oxidative decarboxylation of glycine to CO_2, NH_3, NADH, and methylenetetrahydrofolate during photorespiration, suggests that photorespiration occurs in the lemma and palea. Higher-level constitutive expression of *Cat2* in the lemma/palea is a likely strategy to reduce the buildup of H_2O_2 in the peroxisomes during photorespiration.

Apart from photoassimilates, developing kernels need nitrogen for the biosynthesis of storage proteins. Most of the nitrogen entering the kernel comes from reserves that must be transported to the kernel, although some can be absorbed from the soil (Russell, 1986). In cereals,

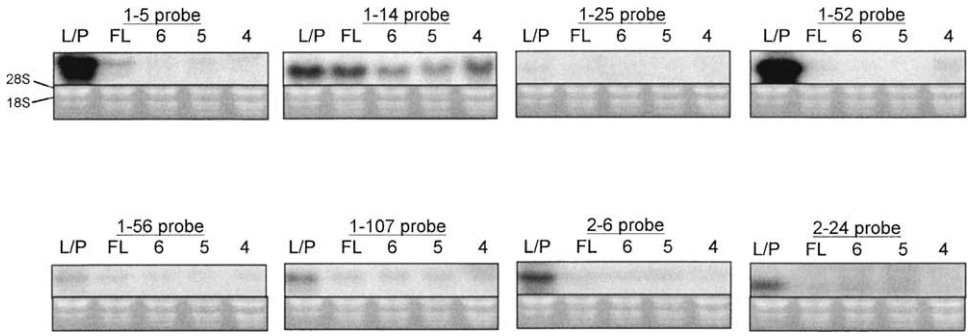


Fig. 4. Expression of selected cloned genes in mature lemma/palea and various leaves of barley. Each lane contained 10 μg total RNA. The RNA blot was hybridized with ^{32}P-labeled 1-5, 1-14, 1-25, 1-52, 1-56, 1-107, 2-6, and 2-24 probes. EtBr stained agarose-formaldehyde gel is included at the bottom of each figure to show equal loading. L/P, pooled lemma/palea RNA from the middle (gelatinous) stage of kernel development; FL, flag leaf; 6, 6th leaf; 5, 5th leaf; 4, 4th leaf.

the predominant amino acid translocated to the kernel is glutamine followed by alanine, asparagine, serine, threonine, and valine (Fisher, 1987). Genes for the biosynthesis of glutamine, glutamate, alanine, and arginine were found in our lemma/palea subtracted libraries (Table 1).

Because of their fibrous nature, the lemma/palea serve as physical barriers to protect florets and developing kernels from pathogens and pests. Greater accumulation of transcripts for structural genes (arabinoxylan arabinofuranohydrolase, proline-rich proteins, caffeoyl CoA *O*-methyl transferase, *S*-adenosyl methionine decarboxylase, and germin/oxalate oxidase) in the lemma/palea than in the flag leaf (Table 1 and Fig. 3) reflects the capacity for continuous deposition of fibrous material in the former, which is important for physical protection. The enzymes caffeoyl CoA *O*-methyl transferase and *S*-adenosyl methionine decarboxylase synthesize lignin via the phenylpropanoid pathway. Proline-rich proteins are structural proteins, which when cross-linked strengthen the cell wall and make it an effective physical barrier against pathogen penetration (Bradley et al., 1992). Higher expression of structural genes in the lemma/palea in the absence of infection maintains the capacity to immediately sequester invading pathogens. Furthermore, cell wall biosynthesis requires H_2O_2 (usually generated by NADPH oxidase) and O_2^- for lignin formation and the oxidative cross-linking of cell wall structural proteins (Bradley et al., 1992; Inz and van Montagu, 1995). Expression of caffeoyl CoA *O*-methyl transferase, *S*-adenosyl methionine decarboxylase, and proline-rich proteins can reduce build up of ROS and cellular damage.

In the absence of pathogen attack, ROS scavenging enzymes expressed in the lemma/palea would function mainly to detoxify H_2O_2 generated during photosynthesis and photorespiration. If the capacity to generate intracellular ROS were kept at low levels by these enzymes, the lemma/palea would require an additional defensive strategy against pathogens. This may be a function of other disease resistance genes expressed in the lemma/palea encoding the jacaline-like protein, lipid transfer protein, ankyrin-like protein, NBS/LRR disease resistance protein, genes for jasmonate biosynthesis, and protease inhibitors (Table 1).

In conclusion, the lemma/palea gene set we identified reflects the unique functions of the lemma/palea as protective structures and sources of carbon and nitrogen to the kernel. This gene set is an excellent resource for cloning tissue-specific gene promoters.

ACKNOWLEDGMENTS

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